

Aus dem Institut für Virologie der Universität Zürich

(Direktor: Prof. Dr. R. Wyler)

Arbeit unter der Leitung von Dr. M. Schwyzer

INDUCTION OF CHEMILUMINESCENCE
IN CULTURED MURINE GLIA CELLS

Inaugural-Dissertation
zur Erlangung der Doktorwürde
der Veterinär-Medizinischen Fakultät
der Universität Zürich

vorgelegt von
Bruno Sonderer
Tierarzt
von Appenzell

Genehmigt auf Antrag von
Prof. Dr. R. Wyler, Referent
Prof. Dr. E. Peterhans, Korreferent

Zürich 1986

Rieker + Partner AG, Glattbrugg



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Summary

Induction of luminol-enhanced chemiluminescence (CL) is studied in glia cell cultures from newborn mice, containing 80 - 90% astrocytes. A burst of CL can be induced by phorbol myristate acetate (TPA), zymosan A, and antibody-coated bovine red blood cells, whereas Sendai virus and several other agents known to induce CL in myeloid cells are ineffective. An optimal CL response is reached at 4×10^5 glia cells/0.75 ml and 70 nM TPA; CL depends on the presence of Ca^{+2} and is not inhibited by sodium azide, suggesting a myeloperoxidase-independent pathway. In parallel experiments, subpopulations of the glia cells are shown to reduce nitroblue tetrazolium and to be capable of phagocytosis. The results suggest that microglia cells and astrocytes are the main contributors to CL. Glia cells of fetal calves are also CL-inducible. The capacity of some glia cells to generate an oxidative burst may reflect their role in immune defense.

Abbreviations

CL	Chemiluminescence
TPA	tumor promoting agent (phorbol 12-myristate 13-acetate)
GFAP	glial fibrillary acidic protein
PMN	polymorphonuclear leucocytes
FCS	fetal calf serum
P/S	Penicillin/Streptomycin
EDTA	ethylenediamine tetraacetic acid
eBBC	embryonic bovine brain cells
HBSS	Hank's balanced salt solution
PBS	phosphate buffered saline
bRBC	bovine red blood cells
NBT	nitroblue tetrazolium (2,2'-Di-p-nitrophenyl- 5,5'-diphenyl- 3,3' [3,3'-dimethoxy- 4,4'-diphenylene] ditetrazolium chloride)
HAU	haemagglutinating units
D'MEM	Dulbecco's modified Eagle medium
LeuOMe	L-leucine methyl ester

Introduction

The brain is considered to be an immunologically privileged organ, as it lacks a lymphatic drainage and is sequestered from the circulatory system by the blood-brain barrier. Recent work has demonstrated, however, that glia cells may be involved in immunological processes in the brain. It has been reported that astrocytes, which can be identified by their specific intracellular protein, the glial fibrillary acidic protein (GFAP), are able to present antigen to T-lymphocytes (Fontana et al. 1984a), to express class II histocompatibility antigens (Carrel et al. 1982) and to secrete an interleukin-1 like molecule (Fontana et al. 1982). Microglia cells, identified by their ability to ingest large amounts of particles, may also participate in immune reactions in the central nervous system, for it has been shown that they express Fc receptors on their surface (Raff et al. 1979).

To assess the biochemical and immunological processes connected with cell activation, a number of techniques are currently used (Volkmann et al. 1984; Pickering et al. 1980). Among these, the measurement of chemiluminescence (CL) is gaining widespread acceptance because it is a technically simple and yet highly sensitive method. In polymorphonuclear leucocytes (PMN), which have been studied best, CL is thought to reflect the generation of highly unstable oxygen species, namely superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\text{OH}^\bullet$) and singlet oxygen ($^1\text{O}_2$). These have the potential to kill engulfed microorganisms and may also participate in cellular cytotoxicity reactions (Trush et al. 1978; Weber and Peterhans 1983). Light is emitted as a result of electronic relaxation and can be enhanced by easily oxidized substrates such as luminol or lucigenin.

The present work demonstrates that cultured murine and bovine glia cells can be induced by various stimuli to emit light. Optimal conditions for CL induction are defined, and attempts are made to identify the cell types that may be most susceptible to CL induction.

Materials and Methods

Preparation of glia cell cultures

a) Glia cells of newborn mice (Fontana et al. 1982):

Newborn, specific-pathogen-free Balb/c mice (9 per preparation, Institute of Animal Husbandry, University of Zurich) were killed by decapitation. The brains were pressed out through the foramen magnum and placed in a Petri dish containing 5 ml Dulbecco's modified Eagle medium (D'MEM; Gibco Europe Ltd, Paisley, Scotland) to remove the residual blood. Then the brains were transferred into fresh D'MEM (5 ml) and weighed. To disaggregate the brain cells and to remove the meninges, brains were transferred with 5 ml D'MEM into a plastic syringe with attached (heat-welded) nylon sieve (210 μm mesh width; Swiss Silk Bolting, Zurich) and passed ten times through the sieve using the syringe plunger. The suspension was mixed with 10 ml of fetal calf serum (FCS) and passed five times through a second syringe with a 132 μm sieve. The brain tissue (90 mg/flask) was seeded in 75 cm^2 tissue culture flasks (Corning Glass Works, Corning, N.Y., USA) with 12 ml D'MEM containing 20% FCS and 0.5% Penicillin/ Streptomycin (P/S). Growth media were replaced on the third day with D'MEM containing 10% FCS, 0.5% P/S and afterwards every seventh day with D'MEM containing 5% FCS, 0.5% P/S. Unless indicated otherwise, the cells were used for the experiments between day 16 and 28 in culture.

Subcultures on coverslips were made in 24-well tissue culture plates (A/S Nunc, Roskilde, Denmark). Monolayers were treated with trypsin/EDTA for 8 minutes, and after washing with D'MEM/10% FCS, 4×10^4 cells per well (1.77 cm^2) were plated in 1 ml D'MEM containing 5% FCS and 0.5% P/S.

b) Embryonic bovine brain cells (eBBC)

Brains were derived from about 3-month-old embryonic calves. The skull was opened under sterile conditions in a laminar flow cabinet. Parts of the cerebrum were plated in a Petri dish containing 5 ml D'MEM. Meninges were removed with forceps. The further handling was the same as described under "glia cells of newborn mice". The inoculation volume was 12 ml per flask and contained 150 mg of suspended cells. Monolayers were established after 16 days in culture.

c) Glioblastoma cell lines:

Monolayers of human glioblastoma cell line cultures were kindly supplied by Dr. N. de Tribolet, Laboratoire de recherche, Service de Neurochirurgie, Lausanne. The cells were passaged 2 - 4 times monthly in D'MEM containing 10% FCS. The following cell lines were assayed: LN18, LN121, LN215 (above passage 80), and LN373, LN375, LN 380 (below passage 5).

Detachment of cells and measurement of CL (Köppel et al., 1984)

Glia cell monolayers were rinsed with 2 ml trypsin/EDTA and then incubated with 5 ml trypsin/EDTA for 8 minutes at 37°C. Trypsinization was stopped with 30 ml of D'MEM containing 10% FCS. Detached cells were centrifuged (10 min, 150 g, 4°C), washed with 5 ml of Ca^{2+} free phosphate buffered saline (PBS) and then suspended at the desired concentration in phenol red-free Hank's balanced salt solution (HBSS) containing 5.6 mM glucose and buffered with 20 mM Hepes, pH 7.2 instead of bicarbonate, and kept on ice. Cell concentration and viability were

determined with a hemocytometer using 0.25% Trypan blue as the diluent. Cell viability ranged between 80 and 90%. To enhance chemiluminescence (CL), 7.5 μ l/ml of 4% (w/v) bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, England) saturated with luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St. Louis, Mo., USA), or 2 μ l of 0.03% (w/v) lucigenin (9,9'-bis (N-Methyl-acridinium nitrate; Boehringer Mannheim GmbH, Mannheim, West-Germany) in PBS were added and 750 μ l of the cell suspensions were dispensed in Pico glass scintillation vials (Packard Instrument Co. Inc., Downers Grove, Ill. USA). Monolayers on coverslips were rinsed twice with D'MEM and once with PBS and then transferred to vials containing 750 μ l of HBSS/glucose containing luminol or lucigenin. The vials were then placed in a water bath (37°C) for 10 minutes (luminol) or 30 minutes (lucigenin) to allow dark adaption of the cells. CL was measured in a modified Kontron model MR 300 DPM liquid scintillation counter (LSC) in the tritium channel with the dead time correction and coincidence units switched off. CL was measured in an automated fashion. Up to 10 samples were placed in a rack and measured repeatedly for 0,1 min periods using a "short circuit" modification of the LSC programm which allowed to return to sample #1 after counting of sample #10. To maintain the temperature of the cells at 37°C, the sample compartement was heated. CL generated by the cells before addition of the stimuli was measured twice. Prestimulation CL is indicated at time = 0 in Fig. 1. To measure light emission of replicate samples at identical time points relative to the addition of stimuli to the cells, the stimuli to be assayed were added in a sequence timed to fit into the progression through the first measurement after the addition of the stimuli to the cells. As a control, one sample was mock-stimulated

with PBS or phorbol (the inactive parent compound of TPA) in dimethyl sulfoxide (DMSO) and run in parallel along with the cells receiving stimuli. Light emission was recorded on line with a HP-85 micro computer and light emission curves were plotted on a HP 7225 A plotter from single point measurement after correcting for dead time losses. The computer programs for on line data acquisition and for plotting and integration of light emission curves relative to the curve of mock-stimulated samples were supplied by Kälin Computer Co., Einsiedeln, Switzerland.

The different stimuli, used at various concentrations, were prepared as follows: phorbol myristate acetate (TPA; Sigma Chemical Co.), Ca ionophore A 23187 and Ionomycin (gifts from Prof. Dr. E. Peterhans, University of Bern, Switzerland) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 0,2 mM and 10 mM, respectively and further diluted with PBS as required. Sendai virus was grown in 11 day old embryonated chicken eggs and purified as described (Peterhans 1980). Zymosan A (Sigma Chemical Co.) was washed three times with 0,15 M NaCl and resuspended in HBSS at a concentration of 5×10^8 particles/ml. In some experiments, zymosan was boiled (10 min in HBSS) and opsonized by preincubation (30 min at 37°C) with human or bovine serum; both, untreated and opsonized zymosan particles were active in CL induction. All these stimuli were kept frozen in small portions at -20°C and were thawed immediately before use.

Bovine red blood cells (bRBC) were obtained by mixing fresh venous blood (40 ml) with 5 ml of sterile PBS, pH 7.2, containing 1.5% (w/v) EDTA. After centrifugation (1.000 g/15 min) the middle third of the packed red cell layer was transferred to a fresh tube and washed twice

with PBS (1.000 g/10 min). To prepare bRBC-antibody complexes, a suspension of bRBC in PBS (10^8 cells/ml) was mixed with an equal volume of purified rabbit immunoglobulins directed against bovine erythrocyte stroma (2.5 mg/ml in PBS; Nordic Immunological Laboratories, Tilburg, The Netherlands), incubated for 1 hr on ice, washed twice with PBS (1.000 g/5 min), resuspended in PBS (10^8 cells/ml), and used on the same day.

Glial fibrillary acidic protein immunofluorescence

Immunofluorescence staining was performed as described (Bignami et al., 1972; Manthorpe et al. 1979). Coverslips were washed twice in D'MEM at 37°C, then quickly immersed vertically in acetone at room temperature for 2 min. Two washes at 37°C in D'MEM and one in PBS were followed by a 30 min incubation at 37°C in air with 40 ul of a 1:50 dilution in PBS of either anti-human GFAP (Dako Corporation Santa Barbara, CA, USA) or normal rabbit serum (Nordic Immunological Laboratories). After three 5 min washes at room temperature in PBS the coverslips were treated at 37°C for 30 min with 40 ul of FITC-conjugated swine anti-rabbit IgG (1:20 dilution in PBS; Nordic Immunological Laboratories). The specimens were then washed three times (5 min each at room temperature) and mounted on a glass slide, sample surface down, in a drop of 9:1 (v/v) glycerol/PBS. Cells were examined for specific FITC fluorescence using a high pressure mercury vapour lamp as light source. The total number of cells per coverslip remained constant throughout this procedure (Manthorpe et al. 1979).

Nitroblue tetrazolium dye reduction assay

Growth medium of glia cells on coverslips was replaced with 1 ml HBSS. One ml of 0.2% (w/v) nitroblue tetrazolium (NBT; Sigma Chemical Co.) in HBSS and 25 nM TPA were added for 15 min at 37°C. Then the coverslips were dipped in HBSS and mounted on a glass slide, sample surface down, in a drop of 9:1 (v/v) glycerol/PBS or stained as described under "GFAP immunofluorescence".

Electron microscopy: Uptake of latex and zymosan by cultured glia cells

Monolayers of murine glia cells in 25 cm² culture flasks were incubated for 2 or 12 hours at 37°C with 5×10^7 particles of opsonized zymosan (see above) or 5×10^7 latex beads (of 1.1 μ m diameter, washed three times in PBS before use; Sigma Chemical Co.) in 5 ml D'MEM containing 5% FCS. After three washes with D'MEM/5% FCS to remove nonadherent particles, the incubation was continued in fresh medium for 60 minutes at 37°C to allow ingestion of residual adherent particles. Cells were then detached by trypsin/EDTA and centrifuged (150 g/10 min). The pellets were fixed with 2.5% glutaraldehyde in 0.1 M s-collidine, pH 7.4, and postfixed with 1% OsO₄ in 0.1 M s-collidine for 1 hr at 4°C. The fixed cell pellets were dehydrated in a graded ethanol series at room temperature and embedded in Epon 812. Semithin sections were stained with methylene blue. Ultrathin sections were contrasted by Mg-uranylacetate and lead citrate and examined with a Philips EM 201.

Results

Induction of chemiluminescence (CL) in murine glia cells

CL kinetics: Brain cells of newborn mice were disaggregated and grown in primary cultures under conditions favouring proliferation of glia cells. In the work described below, we shall call these cultures "glia cells" although a number of nonglial cells may also be present. After 2-4 weeks in culture, the cells were detached with trypsin/EDTA, suspended in HBSS containing glucose and luminol, and then tested for induction of CL using TPA or opsonized zymosan as stimulating agents. Fig. 1 shows the kinetics of luminol-enhanced CL induction in 4×10^5 glia cells at different TPA concentrations. At optimal TPA (70 nM) the CL response reached a peak of 10^6 cpm within 6 minutes and then declined over the next half hour. At the lowest TPA concentration still inducing a significant CL response (8,75 nM), peak CL appeared with a delay of 2-3 minutes and reached 250.000 cpm above background levels of unstimulated controls. A noteworthy difference is seen in the kinetics of TPA-stimulated glia cells (Fig. 1) and cells tested in earlier experiments, e.g. peritoneal macrophages (G. Bertoni and B. Sonderer, unpublished): the CL response in the latter is much slower and extends over a longer time period.

Optimal TPA concentration: Fig. 2 summarizes the data from ten similar experiments, which were performed to evaluate the effect of TPA concentration ranging from 4 nM to 2 μ M on CL. Cells were derived from several independent cultures and exhibited considerable variation in CL peak height. The results were less subject to variation when they were expressed as counts accumulated over a period of

48 minutes as shown. In the range of 4 nM to 35 nM TPA, the CL response increased linearly with the logarithm of the TPA concentration. An optimal burst of CL was induced with TPA at a concentration of 70 nM, whereas higher concentrations were inhibitory.

Optimal cell number: To investigate the dependence of TPA-induced light emission on cell number, ten experiments with cells derived from different cultures were done. Fig. 3 shows that 50.000 cells/vial were sufficient to generate a significant CL response. In the range of 20.000 to 400.000 cells, plotted logarithmically, an approximately linear increase in CL response was observed. Optimal cell number was 300.000 to 400.000 cells and was used in subsequent experiments; higher cell numbers were inhibitory.

Zymosan: Zymosan was used as a stimulus in the experiment shown in Fig. 4. In comparison to the kinetics induced by TPA (Fig. 1), light emission after stimulation with zymosan was characterized by a slower onset and longer duration. Peak response remained below 200.000 cpm with optimal amounts of zymosan (10^7 particles/vial) and was attained within 20-25 minutes.

Subcultures and cell detachment

Recently it was reported that exposing macrophages to proteolytic enzymes before stimulating with TPA or zymosan enhanced their release of oxygen metabolites (Speer et al. 1984). To exclude a possible effect of trypsinization, cells were detached with EDTA alone in some experiments; they exhibited a similar CL response (not shown). Furthermore we subcultured glia cells on small round

coverslips (8 mm diameter), which were then placed in scintillation vials for direct CL measurement of undetached cells. The result (Fig. 5) suggests that trypsin treatment did not enhance CL response. Fig. 5 also demonstrates that CL inducibility is still found in cells after one passage. However, several experiments with detached subcultured glia cells indicated that CL, induced by either TPA or zymosan, was significantly lower than CL observed in primary cultures (not shown).

Fc receptors

It has been shown that up to 2-3% of the cells in central neural cultures of newborn rats are macrophages or macrophage-like cells expressing Fc receptors on their surface (Raff et al. 1979). To investigate whether a CL response could be induced in murine glia cells via Fc receptors, bovine red blood cells (bRBC) coated with rabbit antibody against bRBC stroma were used as a stimulus. Coating is essential, because binding to its target antigen changes the antibody molecule in a way that enables the antibody Fc portion to tightly bind to Fc receptors (reviewed in Weber and Peterhans 1982) and to induce the production of reactive oxygen species in the phagocytic cell (Peterhans et al. 1984). Fig. 6 demonstrates that bRBC or antibody alone failed to induce CL, whereas the addition of antibody-coated bRBC resulted in a burst of CL, indicating the binding of the modulated Fc portions with the corresponding receptors on the cell membranes.

Other stimuli

Sendai virus, at concentrations between 256 and 4096 haemagglutinating units (HAU) per vial, phytohaemagglutinin (10 µg/vial; Wellcome Research Laboratories, Beckenham, England), Lipopolysaccharide (30 µg/vial, Sigma Chemical Co.) and Ionomycin (1 µM to 10 µM) did not induce CL. Ca ionophore A23187 produced inconsistent results: CL was triggered only in one of ten experiments, using the Ca ionophore at concentrations between 1 and 10 µM.

CL of embryonic bovine brain cells (eBBC)

Luminol-enhanced CL-inducibility was also investigated in glia cell cultures of embryonic bovine brains (eBBC) with TPA and zymosan. Fig. 7 shows CL generated in 4×10^5 eBBC after addition of TPA (280 nM). In contrast to the murine cells, a biphasic curve was obtained with peaks at about 12 and 50 minutes. If the dark adaption time was prolonged by 30 min, only the latter peak remained; the kinetics were then similar to those exhibited by TPA-stimulated PMN (not shown).

Stimulation of eBBC with 2×10^6 zymosan particles (Fig. 8) was more effective than stimulation of murine glia cells, but the kinetics were similar.

CL could also be induced in subcultured eBBC. Sendai virus in concentrations ranging from 256 HAU to 2048 HAU failed to induce CL in eBBC.

Biochemical aspects of the CL response

Ca²⁺-requirement: To investigate the Ca²⁺-dependence of TPA induced luminol-enhanced CL, primary murine glia cells were suspended in HBSS lacking Ca²⁺ and stimulated with TPA (70 nM). As seen in Table I, the omission of Ca²⁺ did not decrease the CL response, but when EGTA (2 mM) was included in the medium to chelate residual Ca²⁺, light emission was inhibited by 95%. Most of the CL-response could be restored by removing EGTA and adding back Ca²⁺. Thus, the CL response of murine glia cells requires the presence of Ca²⁺.

Table I: Ca²⁺-requirement of TPA induced CL in murine glia cells

EGTA ¹⁾ (mM)	Ca ²⁺ (mM)	CL response ²⁾	
		10 ⁵ x counts	% of control
-	1,25	75	100
-	-	81	108
2	-	4	5,3
2 ³⁾	1,25 ³⁾	47	62,6

- 1) EGTA was added 10 minutes before CL measurement was started
- 2) the light emission curves exhibited similar kinetics and were integrated over 48 minutes
- 3) cells preincubated with EGTA for 10 minutes at 37°C in Ca²⁺ free HBSS, centrifuged and resuspended in assay medium containing Ca²⁺

Lucigenin: Several experiments were performed with murine glia cells using lucigenin as an amplifier instead of luminol. Lucigenin-dependent CL is thought to reflect O_2^- -generation, whereas luminol enhanced CL is probably triggered by a myeloperoxidase dependent oxidant (Grob and Peterhans 1984). In the subsequent experiments lucigenin was used at a concentration of 2 μM , because preliminary titrations had shown that reasonable background levels were reached within 30 min of dark adaption time under this condition. Cell concentration was about 4×10^5 cells/vial.

In comparison with the luminol enhanced CL, the kinetics of TPA induced light emission curves (Fig. 9) showed a slower onset and a longer duration of peak CL in the presence of lucigenin, reaching similar levels of cpm above mock-stimulated controls (compare with Fig. 1). Zymosan exhibited with lucigenin a very pronounced CL response (Fig. 11) peaking at a level ten times higher than in the luminol amplified system, whereas the kinetics with both amplifiers were similar (compare with Fig. 4). Lucigenin enhanced CL could also be induced by antibody-coated bRBC (not shown). Whereas Sendai-virus and Ionomycin were ineffective, the Ca ionophore A 23187 produced inconsistent results.

Inhibition of myeloperoxidase: The biochemical origin of CL differs according to the nature of the stimulus as well as to the type of cell. A myeloperoxidase-dependent pathway of CL induction, which can be inhibited by sodium azide, has been suggested for PMN stimulated with zymosan (Trush et al. 1978; Weber 1982). Confirming these results, Fig. 11 shows that 10 μM sodium azide reduced CL by more than 50% in human PMN, stimulated with either zymosan, antibody-coated bRBC or TPA. At 100 μM , CL was inhibited more than 90%.

The same experiments were performed with murine glia cells and are also shown in Fig. 11. Contrary to PMN, the CL response of glia cells was insensitive to 100 μ M sodium azide. Only an increase to 500 μ M sodium azide caused any significant inhibition of the CL response; this effect was probably nonspecific as curves of a similar shape were obtained when lucigenin, a myeloperoxidase-independent amplifier, was used instead of luminol (not shown). When cells were preincubated with 500 μ M sodium azide for 10 min, centrifuged and resuspended in fresh assay medium lacking sodium azide, CL returned to the levels of untreated control cells, indicating that 500 μ M sodium azide had no toxic effect. The results suggest that, in glia cells, myeloperoxidase is not essentially involved in the generation of luminol-enhanced CL induced by TPA, zymosan or antibody-coated bRBC.

Attempts to identify the CL producing cells

The measurement of CL indicates the cell's capacity to generate unstable oxygen species (Weber 1982), but it does not allow conclusions as to the cell type that might be responsible for light emission. Although the glia cell cultures examined here consisted of 80-90% astrocytes (see below), the cell population was not homogenous, and the remainder may have included oligodendrocytes, microglia, fibroblasts and neurons.

In a first attempt to attribute the observed CL response to a specific cell type, we tested some well characterized human glioblastoma cell lines of astrocytic origin (Fontana et al. 1984b). All glioblastoma cell lines mentioned under "Materials and Methods" were screened for luminol- and lucigenin-enhanced CL with TPA, opsonized zymosan, Ca ionophore and Sendai virus. None of these treatments induced a significant CL response, suggesting either that astrocytes are not CL responders or that these cell lines had lost CL responsiveness in culture.

Another possibility to be considered is that CL in cultured glia cells might be produced by contaminating cells from outside the central nervous system, the most likely source being blood monocytes or polymorphonuclear leucocytes which are excellent CL responders. The following observations argue against this possibility. CL was measured in glia cells between day 0 and 20 in culture. Cells in 75 cm² flasks were detached, split in ten equal volumes and CL was measured. No TPA-induced CL emission could be detected in the dissociated brain cells just before seeding; a control experiment with added neutrophils (not shown) demonstrated that the absence of CL was not due to quenching by the brain cells. Light emission strongly increased between day 2 and 16, indicating that cell proliferation or differentiation in culture was required for CL. After day 16 no significant increment on CL was observed (Fig. 12). This observation excludes PMN as a possible source of CL.

Mononuclear phagocytes adhere fast and tightly to plastic surfaces, whereas glia cells show relatively low adhesiveness (Fontana et al. 1984b; Manthorpe et al. 1979). To test the adhesiveness of potentially CL-positive cells, glia cell cultures were prepared and seeded in plastic culture flasks as described. After two hours at 37°C the culture supernatant was gently aspirated and transferred to another culture flask; the adherent cells remaining in the flask were covered with 12 ml of fresh medium. After 16 days in culture confluent monolayers were established in both flasks. Cells were detached with trypsin/EDTA and addition of TPA to 4×10^5 cells evoked a similar CL response in both (not shown).

It was reported recently that L-leucine methyl ester (Leu-OMe) causes selective death of human monocytes by lysosomal disruption (Thiele et al. 1983). Glia cells in culture were preincubated for 40 minutes with 2 mM Leu-OMe in D'MEM. Then cells were rinsed twice with D'MEM and fresh growth medium was added. After 20 hours in culture, cells were detached and stimulated with TPA. CL response was not reduced compared with untreated controls. In a second experiment, Leu-OMe was added to detached cells in HBSS/glucose/luminol; 40 min later TPA was added, and CL was measured over 48 min. Compared to a control without Leu-OMe, CL was reduced by 40%. In comparison, the same treatment blocked CL in murine peritoneal macrophages by more than 80%. These results again tend to exclude blood monocytes as a source of CL in these glia cell preparations.

Microscopic examination

As a further step in the attempts to identify the cells, which might produce CL, we used the NBT dye reduction assay. The reduction of NBT in activated phagocytic cells, visible by forming blue purple granules of formazan, indicates the enhanced O_2 metabolism within these cells after stimulation (Volkman et al. 1984). Murine glia cells on coverslips were incubated in HBSS containing NBT, and stimulated with TPA. Approximately 10% of the cells stained intensely blue. They often seemed to be on top of the monolayers in small groups of five to ten cells (Fig. 13B). Astrocytes were identified after acetone fixation by their characteristic fibrillary staining with anti-GFAP antibody (Raff et al. 1979; Stieg et al. 1980; Fig. 13A). When monolayers, preincubated with NBT, were stained by the indirect immunofluorescence technique with anti-GFAP serum, between 80 and 90% of all cells were GFAP positive; Fig. 13C and D show GFAP positive cells that reduced NBT, but the majority of NBT-positive cells did not label with anti-GFAP serum.

To investigate the phagocytic activities, glia cell cultures were exposed to latex beads or zymosan particles for two or 12 hours (Taffet and Russell, 1981). Cells on coverslips were screened light microscopically for ingested particles. Although we found cells with particles associated with their processes and cell bodies, we could not decide whether these were ingested or simply adherent. This difficulty could be eluded by making semithin sections (not shown) of cells, which were detached, centrifuged and fixed after incubation. To give an estimate of the percentage of phagocytic cells in glia cell cultures, cells in eight random areas on the semithin sections were examined for ingested particles and counted (between 500 and 750 cells per section). After two hours of incubation, 2-3% of the cells had ingested large numbers of either latex or zymosan. In the cultures exposed to latex beads, we found additional cells which had ingested only a few particles (about 3%). When cells were incubated for 12 hours, the percentage of cells, which were abounding with particles, remained the same. The portion of cells with a few engulfed latex beads was increased by 5 times (16%), and about 4% of the cells had ingested additional zymosan particles.

In the electron microscope, a majority of the cells could be identified as astrocytes containing numerous bundles of intermediate filaments; some of these had engulfed latex beads (Fig. 14A) or zymosan particles (Fig. 14B and C).

Discussion

This work demonstrates, to our knowledge for the first time, that cells of the central neural system are able to generate CL. Murine glia cells exhibit CL upon stimulation with such widely different agents as TPA, zymosan, or antibody-coated bRBC. The measurement of CL is an established technique for studying events associated with phagocytosis and the generation of highly unstable oxygen species (H_2O_2 , $\cdot\text{O}_2$, $\cdot\text{OH}$, $^1\text{O}_2$) in myeloid cells (Trush et al. 1978). The present results suggest that in glia cells, too, phagocytosis and increased oxygen metabolism is accompanied by CL-inducibility.

Glia cell cultures were prepared under conditions favouring the growth of astrocytes (Fontana et al. 1982; Raff and Miller 1983). Determined by GFAP immunofluorescence, our cultures contained 80-90% astrocytes in agreement with results by others (Raff et al. 1979; Manthorpe et al. 1979). That such cultures also contain oligodendrocytes and microglia has been amply documented (Raff et al. 1979; Raff and Miller 1983); in addition we observed the presence of ependymal cells by electronmicroscopy (M. Vandeveld, P. Wild, B. Sonderer, unpublished results). Our results show that some astrocytes, although a minority, exhibit increased oxygen metabolism detected by NBT dye reduction assay, and that they ingest zymosan or latex particles shown by electron microscopy. In addition GFAP-negative cells capable of reducing NBT were observed to lie on top of the astrocyte monolayer in an arrangement typical of microglia cells. The proportion of glia cells ingesting large amounts of particles was about 3% shown by light microscopy; a similar percentage of microglia has

been found in glia cell cultures derived from newborn rats (Raff et al. 1979). Thus, both astrocytes and microglia cells can be considered as potential CL responders. In collaboration with K. Frei and A. Fontana we have recently been able to separate microglia from astrocytes by a mechanical method; both cell types, cultured separately, exhibited about equal CL inducibility (K. Frei, A. Fontana, B. Sonderer, unpublished). The use of a cell sorter to separate the different types of cells would help to clarify the exact origin of CL induced by the various stimuli.

Comparison of myeloid cells, so far studied best, and glia cells reveals similarities and differences in the CL response. Although both cell types exhibit similar CL kinetics, higher numbers of glia cells are required for an optimal CL response, probably because the proportion of phagocytic cells is smaller. Glia cells respond to fewer different stimuli than do myeloid cells; e.g. Sendai virus, a potent CL-inducer in PMN and macrophages, has no effect on glia cells, possibly due to a lack of virus receptors. However, both cell types can be stimulated by tumor-promoting phorbol esters (TPA), and the presence of Ca^{2+} is required for the CL response. Recently, the role of phosphatidyl inositol metabolism in signal transmission across cell membranes has been reviewed (Joseph 1985; Majerus et al., 1985). The key elements appear to be inositol trisphosphate, which triggers the mobilisation of Ca^{2+} from internal stores, and diacylglycerol, which activates protein kinase C, a crucial enzyme in the stimulatory cascade. TPA structurally resembles diacylglycerol but is a more potent activator of protein kinase C because it is not degraded as rapidly.

Induction of CL by complexed or surface bound antibody depends on the presence of Fc receptors in the cell membrane (Weber and Peterhans 1982). Our finding that CL is induced in glia cells by antibody-coated bRBC correlates well with the demonstrated presence of Fc receptors on microglia cells (Raff et al. 1979). For zymosan, receptor-independent as well as mannose glycoprotein receptor-dependent stimulatory pathways appear to be possible (Leoni and Dean 1984). For all stimuli tested, we found that CL of glia cells could be amplified by either luminol or lucigenin. Whereas luminol-enhanced CL in human PMN reflects the generation of a myeloperoxidase-dependent oxidant (Grob and Peterhans 1984), this does not seem to be the case in glia cells.

The brain is normally isolated from invading viruses and microorganisms, as well as from the body's immune system, by the blood-brain barrier. When infection does occur, however, the brain is capable of mounting a specific immune response, which is thought to comprise secretion of γ -interferon by infiltrating T-cells, induction of histocompatibility antigens on brain cells by γ -interferon (Wong et al. 1985), and presentation of foreign antigens by astrocytes (Fierz et al. 1985). These immunological processes prepare the ground for the destruction of infected brain cells by effector cells originating from outside the brain, i.e. by cytotoxic T-cells or natural killer cells. The present observations point to the possibility that brain cells by themselves (i.e. astrocytes and microglia cells) may play a direct role as effector cells by generating an oxidative burst. Indeed, it may be appropriate for the brain to mount its own first line of defense before calling in potentially harmful

T-cells. On the other hand reactive oxygen species may in some instances contribute to brain damage such as demyelination or the generation of autoimmune reactions. The measurement of CL, a simple and sensitive technique, should help to test these hypotheses.

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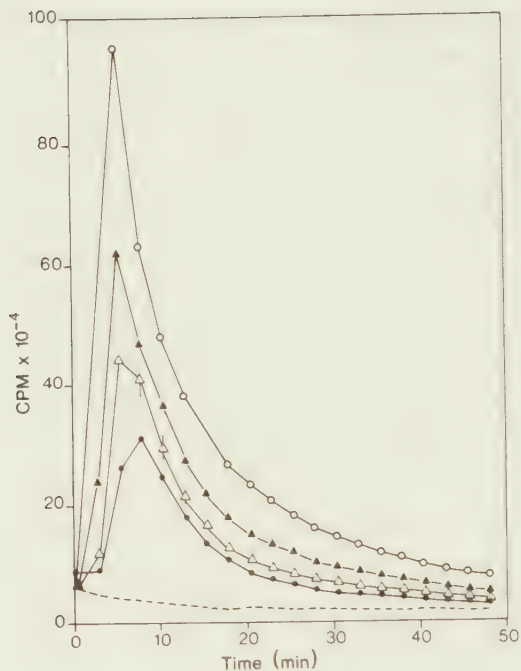


Fig. 1 Induction of luminol-enhanced CL in murine glia cells by TPA:

Each vial contained 4×10^5 glia cells suspended in 0.75 ml HBSS/glucose/luminol. After adding different amounts of TPA, the CL response was recorded as described in "Materials and Methods". Samples were run in duplicate, bars represent standard deviation. Where no bar appears, standard deviations is smaller than the symbol.

75 nM TPA (O-O); 35 nM TPA (▲-▲); 17,5 nM TPA (Δ-Δ); 8,75 nM TPA (●-●); mock-stimulated control (PBS, ---)

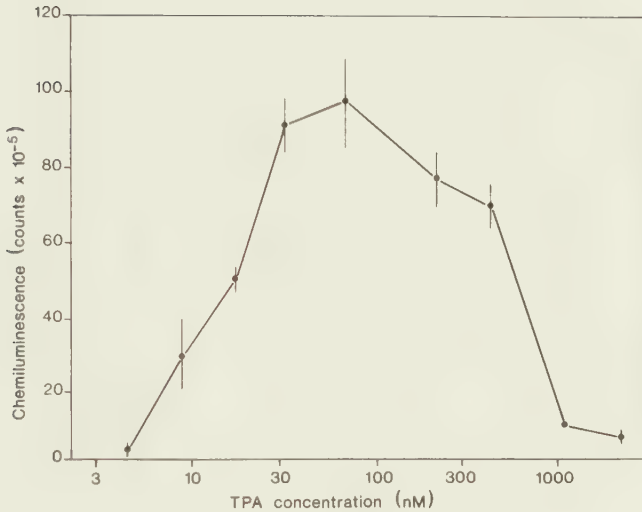


Fig. 2 Effect of TPA concentration on CL induction in murine glia cells:
CL was measured in 4×10^5 cells after stimulation with various concentrations of TPA. Points represent counts accumulated in the 48 minute period following stimulation, calculated from the areas under the light emission curves. The data are from ten experiments like Fig. 1, and bars represent standard error about the mean (SEM).

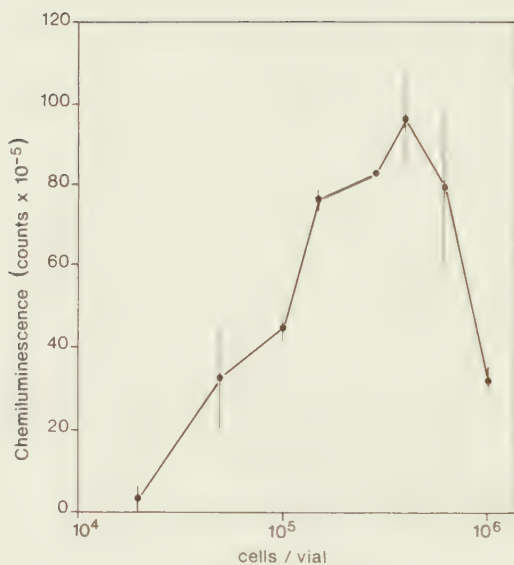


Fig. 3 Dependence of TPA induced CL on cell concentration:

Murine glia cells were suspended in HBSS/glucose/luminol at different concentrations and stimulated with 70 nM TPA/vial. Points represent counts accumulated over a period of 48 minutes following stimulation (determined as in Fig. 2).

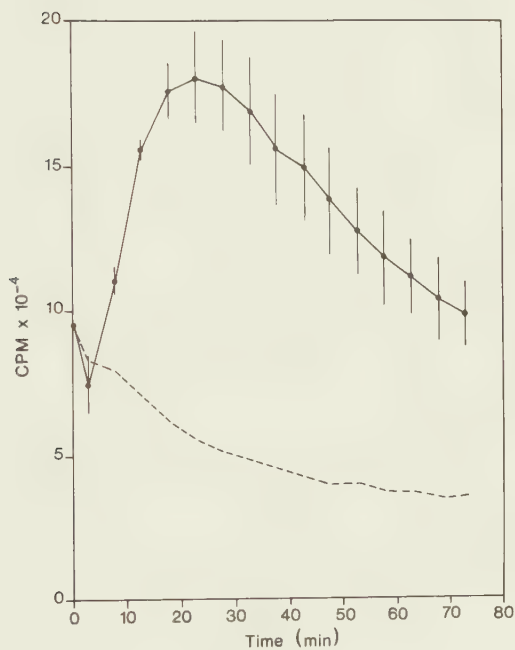


Fig. 4 Induction of luminol-enhanced CL by opsonized zymosan:
CL was measured in 4×10^5 murine glia cells after stimulation with 10^7 particles of zymosan (●-●) or PBS (---).



Fig. 5 Induction of CL in undetached, subcultured murine glia cells by TPA:

A coverslip bearing 27.000 cells was placed in 750 μ l HBSS/glucose/luminol and stimulated with 70 nM TPA (●-●) or PBS (---).

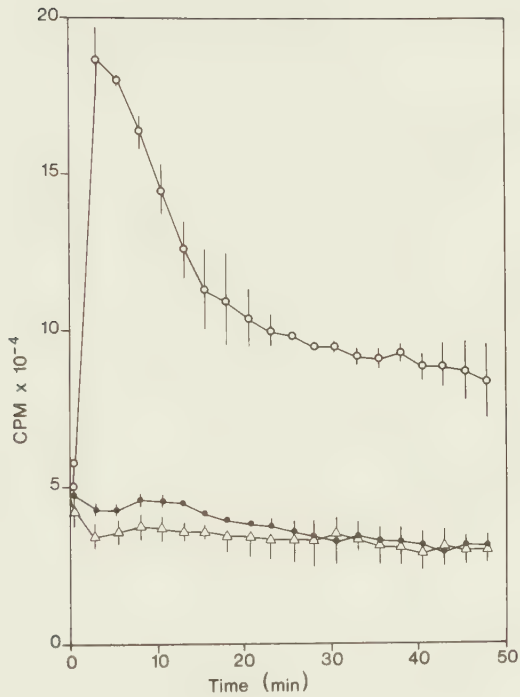


Fig. 6 Induction of luminol-enhanced CL by antibody coated bRBC:
CL was measured in 4×10^5 murine glia cells after addition of 2×10^6 antibody-coated bRBC (O-O) or 25 μ g of antibody alone (●-●) or 2×10^6 uncoated bRBC (Δ - Δ)

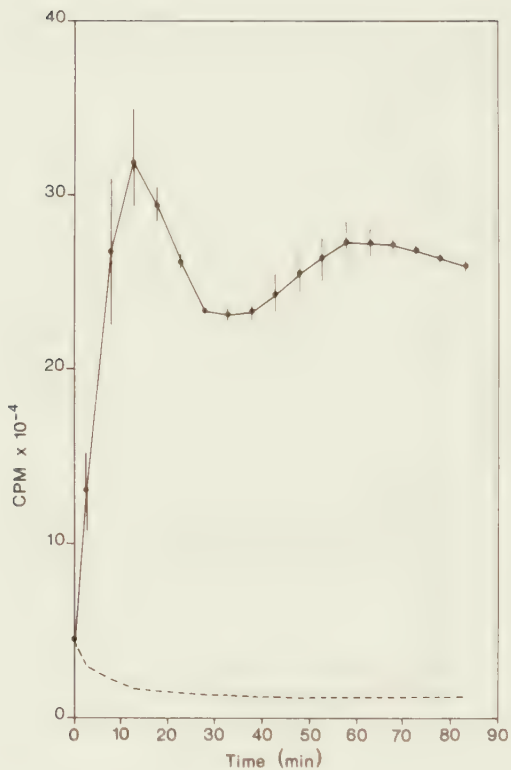


Fig. 7 Induction of CL in embryonic bovine brain cells by TPA:

CL was measured in 4×10^5 eBBC after stimulation with 280 nM TPA (●-●) or PBS (---)

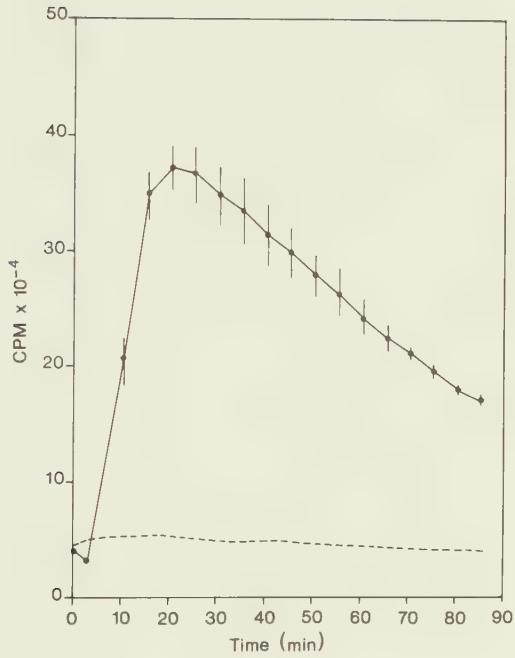


Fig. 8 Induction of CL in eBBC by opsonized zymosan: CL was measured in 4×10^5 eBBC after stimulation with 10^7 particles of zymosan (●-●) or PBS (---)

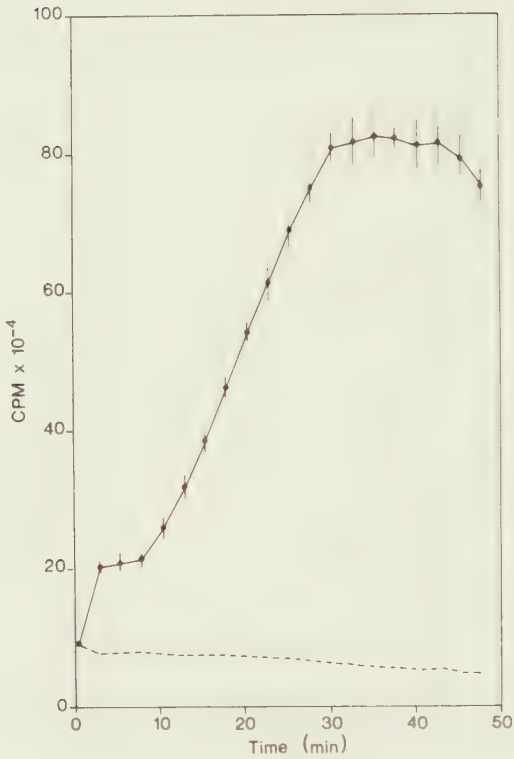


Fig. 9 Lucigenin-enhanced CL induction by TPA in murine glia cells:
 4×10^5 cells were suspended in HBSS/glucose/
lucigenin and stimulated with 70 nM TPA (●-●) or
PBS (---)

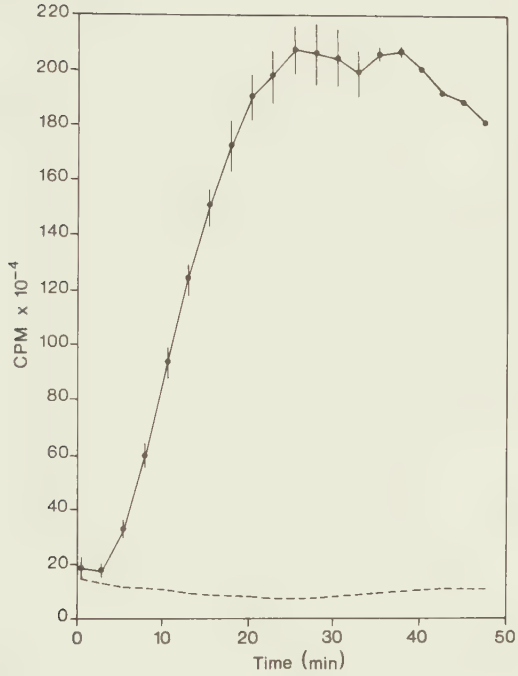
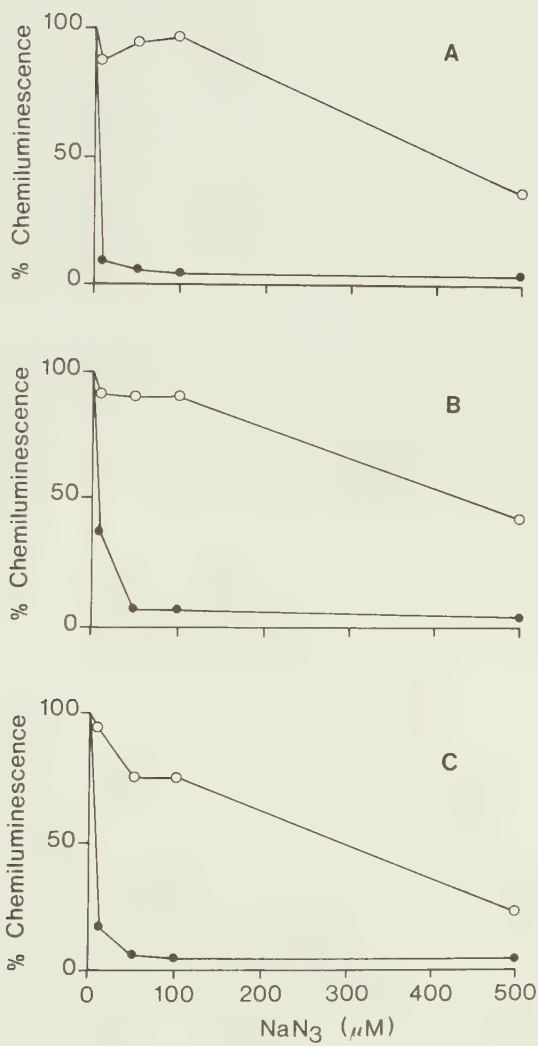


Fig. 10 .Lucigenin-enhanced CL induction by opsonized zymosan:
To 4×10^5 cells were added 10^7 zymosan particles (●-●) or PBS (---)

Fig. 11 Comparison of the effect of sodium azide in CL response of human PMN and murine glia cells: To $7,5 \times 10^4$ PMN (●-●) or 4×10^5 glia cells (○-○) were added TPA (4 nM to PMN; 70 nM to glia cells) (A); 10^7 particles of opsonized zymosan (B); or 10^6 antibody-coated bRBC (C). Sodium azide (10,50,100,500 μ M) was added 10 min before stimuli. Points represent counts accumulated over a period of 30 min following stimulation and are expressed in % CL of untreated controls.



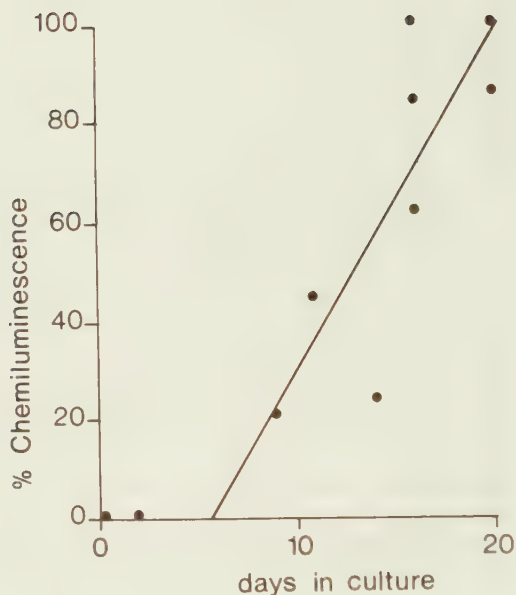


Fig. 12 CL of murine glia cells after different times in culture:

Cells were detached between day 9 and 20 in culture, centrifuged, resuspended and divided in 10 equal volumes. CL, induced by 70 nM TPA, was compared. Points represent counts over a period of 48 min following stimulation and are expressed in % CL of the maximum response.

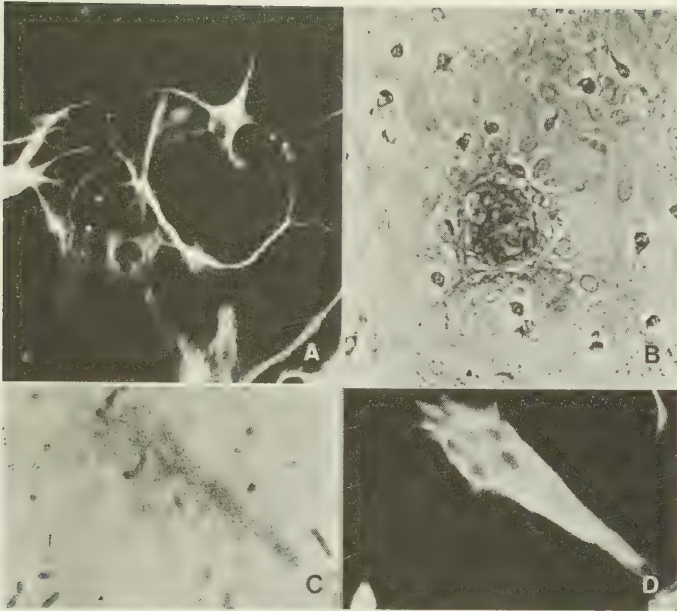


Fig. 13 GFAP staining and NBT reduction:

- A: Cultured murine glia cells on coverslips, fixed in acetone and labelled with rabbit anti-GFAP followed by FITC-conjugated swine anti rabbit IgG. Note the typical fibrillary staining of the astrocytes.
- B: Glia cells incubated with NBT and stimulated with TPA. Note the group of NBT positive cells on top of the monolayer.
- C;D Examples of NBT reducing astrocytes. NBT dye reduction was performed as described (C). Then cells were fixed in acetone and labelled with rabbit anti-GFAP by the indirect immunofluorescence technique (D). Note the small granules of formazan in (C).

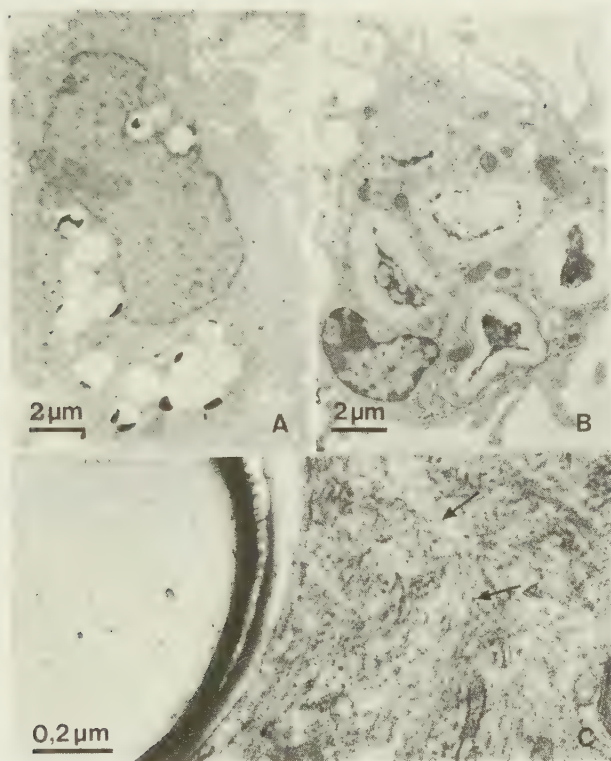


Fig. 14 Phagocytic ingestion of latex beads (A) or zymosan particles (B,C) by murine astrocytes: glia cells were incubated for 2h (B) or 12h (A,C) with a suspension of particles and then washed, trypsinized and examined electron microscopally as described in "Materials and Methods". A and B show entire astrocytes with ingested particles at 6.000 x magnification; C shows a detail at 87.000 x magnification of a comparable cell to that shown in A, exhibiting the intermediate filaments typical for astrocytes (arrows).

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